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Connection of *Gnomonia intermedia* to *Discula betulina* and its relationship to other taxa in *Gnomoniaceae*[☆]

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ABSTRACT

Discula betulina is a foliar pathogen on birch (*Betula*) and *Gnomonia intermedia* is found on overwintered birch leaves. Perithecia of *G. intermedia* developed *in vitro* on colonies of *D. betulina* isolated from birch tissues in late summer, and single ascospores of *G. intermedia* consistently developed into colonies similar to *D. betulina*, producing typical *D. betulina* conidia. Isolates of *D. betulina* could be grouped into two mating types, which produced fertile perithecia of *G. intermedia* when mated with each other. Mycelia from single-ascospore and single-conidial isolates were inoculated onto shoots of downy birch, causing lesions and die-back from which *D. betulina* was consistently isolated. ITS region ribosomal DNA sequence analysis confirmed the results of the morphological and mating studies, and found that the closest known relatives of *G. intermedia*/*D. betulina* are *Gnomoniella nana* and *Sirococcus clavignenti-juglandacearum*. The conclusion from these studies is that *D. betulina* is the anamorph of *G. intermedia*.

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Introduction

Discula betulina (syn. *Gloeosporium betulinum*, *Myxosporium devastans*) is a common foliar pathogen of birch (*Betula* spp.) in the UK, continental Europe and North America, causing leaf spots that can lead to premature defoliation (Phillips & Burdekin 1982; Sinclair *et al.* 1987). The fungus is frequently observed on young, planted birch in Scotland, together with the other leaf spot fungi, *Marssonina betulae* (Green 2004) and *Septoria betulae* (Green 2005a). *Discula betulina* forms brown lesions with dark margins on both sides of the leaves, but fruits predominantly on the undersides. Although generally regarded as a leaf disease, *D. betulina* has also been found on diseased and healthy shoots of birch in Scotland (Green 2004), and

caused die-back of young shoots when inoculated onto birch saplings (Paetzholt & Schneider 1966; Green 2004). It is thought that *D. betulina* may contribute to the widespread die-back of young birch recently reported in Scotland, hence the increased recent interest in the biology of this fungus (Green 2004, 2005b).

Currently, little is known about the life cycle of *D. betulina*, and a sexual state has not yet been reported (Sinclair *et al.* 1987). When isolations were made from shoots of silver birch (*Betula pendula*) in Scotland during late summer, perithecia belonging to a species of *Gnomonia* developed on culture plates containing colonies of *D. betulina* (Green 2004). Based on the morphology of the perithecia and ascospores, this fungus was identified as *G. intermedia* by the Centraalbureau voor Schimmelcultures

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(CBS), Utrecht, The Netherlands. *G. intermedia* has been described from overwintered leaves of birch in Europe and North America (Barr 1978) but an association with *D. betulina* has not been made.

This paper reports on studies undertaken to test the hypothesis that *D. betulina* is the anamorph of *G. intermedia*. These include cultural, morphological and life-cycle studies that link the two fungal states. Isolates of *D. betulina* and *G. intermedia* were mated to assess sexual compatibility, and inoculated onto birch to compare pathogenicity and to satisfy Koch's Postulates. Finally, DNA analysis was undertaken to confirm the connection between *D. betulina* and *G. intermedia*, and to determine relatedness to other known members of *Gnomoniaceae*. The molecular data are discussed in terms of their implications for the taxonomy of genera within *Gnomoniaceae*.

Materials and methods

Fungal material, morphology and life cycle

Healthy and diseased young shoots and leaves of silver birch and downy birch (*Betula pubescens*) were collected from various locations in Scotland during August and September 2002 and 2004. Leaf and shoot pieces, approximately 5 mm², were cut from the margin of lesions or from healthy tissue, surface sterilised in 70 % ethanol for 1 min, 5 min in 1.25 % sodium hypochlorite and 30 s in 70 % ethanol. They were then rinsed twice in sterile distilled water, blotted dry on sterile filter paper, plated onto 2 % malt agar and incubated in the dark at room temperature for three to four weeks. Single-ascospore cultures of *Gnomonia intermedia* were obtained by squashing perithecia which developed *in vitro*, spreading the contents onto 2 % malt agar, incubating overnight at room temperature in the dark, and transferring germinated individual ascospores to fresh agar plates using a stereomicroscope. Cultures of *Discula betulina* were obtained by transferring mycelium or individual conidia from isolated colonies also growing *in vitro*. All cultures were grown on 2 % malt agar and incubated at 20/15 °C day/night temperatures with a 12 h photoperiod consisting of cool white fluorescent and near-ultra violet light. The sizes of 10 asci, 20 ascospores, and 20 conidia from each of five isolates of *G. intermedia* or *D. betulina* were measured using a compound microscope containing an eyepiece micrometer, making a total of 50 asci, 100 ascospores and 100 conidia measured.

To determine whether the teleomorph state is formed over winter, leaves of silver birch and downy birch, which were naturally infected with *D. betulina*, were placed on either wet or dry filter paper in plastic Petri dishes and maintained inside a cooled incubator at 4 °C with continuous darkness. Leaves maintained on the wet filter paper were misted lightly with distilled water at weekly intervals. Diseased leaves were also placed in 15 cm² nylon mesh bags and maintained outside on the ground. The study was set up in September 2004, and the leaves examined for formation of ascocarps in April 2005. Single ascospore cultures were obtained from fertile perithecia that developed on overwintered leaves.

Isolate crosses

This study was set up in October 2004 to test the ability of 20 isolates of *Discula betulina* and one isolate of *Gnomonia*

intermedia (Table 1) to form fertile perithecia when mated. Pair wise crosses using all isolate combinations were performed on Petri plates containing 1 % water agar, with an autoclaved flat birch toothpick placed across the centre of each plate to provide a substrate for the formation of perithecia. Mycelial plugs of the two parental strains were placed on the agar surface, one either side of the toothpick. Inoculated Petri plates were incubated at room temperature in direct, natural light, and examined for the development of perithecia at two, four and six months after inoculation. When perithecia were observed in any pair-wise cross, they were crushed and the contents examined under a compound microscope to confirm the presence of mature ascospores. Single-ascospore isolates were obtained from fertile perithecia which developed during this study.

Pathogenicity tests

Pathogenicity tests were carried out on one-year-old seedlings of Scottish provenance downy birch, which were grown from seed in a greenhouse. Two isolates of *Discula betulina* and two isolates of *Gnomonia intermedia* were inoculated onto four replicate seedlings per isolate using 3 mm diameter mycelial plugs cut from the growing margin of single-conidial and single-ascospore cultures on 2 % malt agar. Four seedlings were inoculated with plugs of sterile 2 % malt agar as controls. The plugs were placed, mycelial side down, onto the expanding leading shoot of each seedling, 2–3 cm from the base of the current year's shoot extension. Shoots were wounded before inoculation by gently scraping back a 10 × 2 mm section of epidermis at the inoculation site using a sterile scalpel. After inoculation, a droplet of sterile distilled water was placed on each plug, and the plugs sealed in place with parafilm. Four mycelial plugs of each isolate were also plated onto 2 % malt agar, incubated in the dark at room temperature for 7 d, and examined for colony development to confirm inoculum viability. Inoculated seedlings were placed outside for 14 d, at which time the parafilm was removed and lesion lengths on the leading shoots measured. Back-isolations were carried out from a lesion on one seedling per isolate, and from the inoculation site on one control seedling, using methodology described above. Four tissue pieces per isolate or control treatment were plated onto 2 % malt agar, incubated in the dark at room temperature for three to four weeks, and colonies identified by morphological characteristics.

DNA analysis

All isolates sequenced were isolated from silver birch or were the result of crosses of isolates from silver birch (Table 1). Genomic DNA was extracted from approximately 50 mg of mycelia scraped from the surface of a 3–5 d old culture growing on Difco potato dextrose agar (PDA). The ITS regions 1 and 2 of the nu-rDNA including the 5.8 S nu-rDNA were amplified using primers ITS 5 and ITS 4 (White *et al.* 1990). Five isolates of *Discula betulina* (CBS 119202 = 2006a, CBS 119193 = 2041a, CBS 119518 = 2043a, CBS 119188 = 2044a, CBS 119352 = 2081) and four isolates of *Gnomonia intermedia* (CBS 119194 = 2048a, CBS 119196 = 2070a, CBS 119192 = 2083b, CBS 119197 = 2087a; Table 1) were sequenced. In addition, the ITS gene region was sequenced for representatives of taxa within the

Table 1 – Origin of isolates of *Discula betulina* and *Gnomonia intermedia* used in isolate crossing studies^a and DNA analysis^b

| Isolate | State | Location of origin in Scotland | Host material | Method of isolation | Date of isolation |
|----------------------|----------------------------|---|-----------------------------|---------------------|-------------------|
| 2004 ^a | <i>Discula betulina</i> | Blair Atholl, Perthshire | <i>Betula pendula</i> shoot | Mycelial transfer | August 2001 |
| 2005a ^a | <i>D. betulina</i> | Pentland Hills, Midlothian | <i>B. pendula</i> shoot | Single conidial | July 2001 |
| 2006a ^{a,b} | <i>D. betulina</i> | Loch Glascarnoch, Ross & Cromarty | <i>B. pendula</i> shoot | Single conidial | July 2001 |
| 2013a ^a | <i>D. betulina</i> | Cornharrow, Dumfriesshire | <i>B. pubescens</i> shoot | Single conidial | August 2001 |
| 2019 ^a | <i>D. betulina</i> | Glen Artney, Perthshire | <i>B. pendula</i> shoot | Mycelial transfer | September 2001 |
| 2024 ^a | <i>D. betulina</i> | Ochil, Perthshire | <i>B. pendula</i> shoot | Mycelial transfer | September 2001 |
| 2032 ^a | <i>D. betulina</i> | Strahanna Woods, Dumfriesshire | <i>B. pendula</i> shoot | Mycelial transfer | May 2002 |
| 2041a ^{a,b} | <i>D. betulina</i> | Caplawhead, Clackmannanshire | <i>B. pendula</i> leaf | Single conidial | June 2003 |
| 2043a ^{a,b} | <i>D. betulina</i> | Glasgow, Lanarkshire | <i>B. pendula</i> leaf | Single conidial | July 2003 |
| 2044a ^{a,b} | <i>D. betulina</i> | Blair Atholl, Perthshire | <i>B. pendula</i> leaf | Single conidial | June 2003 |
| 2048a ^{a,b} | <i>Gnomonia intermedia</i> | Strahanna Woods, Dumfriesshire | <i>B. pendula</i> shoot | Single ascospore | September 2002 |
| 2070a ^b | <i>G. intermedia</i> | Strahanna Woods, Dumfriesshire | <i>B. pendula</i> leaf | Single ascospore | September 2004 |
| 2071 ^a | <i>D. betulina</i> | Strahanna Woods, Dumfriesshire | <i>B. pendula</i> leaf | Mycelial transfer | September 2004 |
| 2072 ^a | <i>D. betulina</i> | Strahanna Woods, Dumfriesshire | <i>B. pubescens</i> leaf | Mycelial transfer | September 2004 |
| 2073 ^a | <i>D. betulina</i> | Strahanna Woods, Dumfriesshire | <i>B. pubescens</i> leaf | Mycelial transfer | September 2004 |
| 2075 ^a | <i>D. betulina</i> | Edderton farm, Ross & Cromarty | <i>B. pendula</i> leaf | Mycelial transfer | August 2004 |
| 2076 ^a | <i>D. betulina</i> | Loch Glascarnoch, Ross & Cromarty | <i>B. pubescens</i> leaf | Mycelial transfer | August 2004 |
| 2077 ^a | <i>D. betulina</i> | Loch Glascarnoch, Ross & Cromarty | <i>B. pubescens</i> leaf | Mycelial transfer | August 2004 |
| 2078 ^a | <i>D. betulina</i> | Glen Rinnes, Banffshire | <i>B. pubescens</i> leaf | Mycelial transfer | August 2004 |
| 2080 ^a | <i>D. betulina</i> | Ulzieside, Dumfriesshire | <i>B. pendula</i> leaf | Mycelial transfer | September 2004 |
| 2081a ^b | <i>D. betulina</i> | Glen Artney, Perthshire | <i>B. pendula</i> leaf | Mycelial transfer | September 2004 |
| 2083b ^b | <i>G. intermedia</i> | Perithecium resulting from in vitro cross between isolates 2081 and 2019 | | Single ascospore | January 2005 |
| 2087a ^b | <i>G. intermedia</i> | Perithecium resulting from in vitro cross between isolates 2071 and 2041a | | Single ascospore | January 2005 |
| GAA15 ^a | <i>D. betulina</i> | Glen Artney, Perthshire | <i>B. pendula</i> leaf | Mycelial transfer | September 2004 |

a Isolate used crossing studies.

b Isolate used DNA analysis.

Gnomoniaceae and *Melanconidaceae* (GenBank accession nos [DQ323523–DQ323544](#)). The LSU of the nu-rDNA was sequenced for *G. intermedia* CBS 119194 (2048a), *Gnomoniella nana* CBS 883.79, *Amphiportha hrancensis* CBS 119289, and *Sirococcus clavignenti-juglandacearum* AR 3791 (GenBank accession nos [DQ323519–DQ323522](#)).

Gene fragments were amplified in 50 µl reactions on a GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA) under the following reaction conditions: 10–15 ng genomic DNA, 200 µM each dNTP, 2.5 units Amplitaq (Applied Biosystems), 25 pmol of each primer, and 10 µl of the supplied 10× PCR buffer, which includes 15 mM magnesium chloride for a final concentration of 1.5 mM magnesium chloride. The thermal cycler programme was: 2 min at 95 °C followed by 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min at 72 °C, with a final extension period of 10 min at 72 °C. Amplified products were sequenced with the BigDye version 3.1 ready reaction kit (Applied Biosystems) on an ABI 3100 automated DNA sequencer (White *et al.* 1990). Raw sequences were edited using Sequencher version 4.5 for Windows (Gene Codes Corporation, Ann Arbor, MI) and are deposited in GenBank as [DQ323519–DQ323544](#). Pair-wise sequence comparisons were done through GenBank with BLASTn (Altschul *et al.* 1990) using default settings and BLAST 2 (Tatusova & Madden 1999) with the following settings differing from the defaults to ensure the entire sequences were aligned: match = 1, mismatch = –1, gap open = 3, and gap extension = 2. Additional

ITS sequences not generated in this study used in the BLAST 2 analysis are as follows: *Gnomonia gnomon* [AY818956](#), *Gnomoniella fraxini* [AY455812](#), *Discula destructiva* [AF429748](#), *S. clavignenti-juglandacearum* [AY437754](#), *S. conigenus* [AY437773](#) and *Discula umbrinella* [AJ293871](#) (as *Fusicoccum quercus*).

Sequence alignment and analyses

LSU and ITS sequences from representative genera in the *Gnomoniaceae* and *Melanconidaceae* with taxa in the *Cryphonectria* complex (Castlebury *et al.* 2002) were manually aligned separately and then concatenated into a single file for analysis. ITS region sequences were not available for taxa in the *Cryphonectria* complex. Alignments were adjusted using GeneDoc 2.6.001 (<http://www.psc.edu/biomed/genedoc/>). The combined alignment was deposited in TreeBASE. Trees were inferred by the NJ method (TrNef + I + G) as determined by Modeltest 3.7 (Posada & Crandall 1998) with I = 0.7539 and G = 0.6782 and by MP using the heuristic search option with the random addition sequence (1000 replications), MULTREES on and the tree bisection–reconnection (TBR) branch swapping option of PAUP 4.0b10 (Swofford 2002). All aligned positions were included in the analysis. All characters were unordered and given equal weight during the analysis. Gaps were treated as missing data. Relative support for branches was estimated with 1000 BS replications (Felsenstein 1985).

with MULTREES on and TBR and ten random sequence additions for the MP BSs.

Results

Fungal material, morphology and life cycle

Discula betulina was frequently isolated from both healthy and diseased shoots and leaves of birch, producing fast-growing colonies (approx. 7–8 cm diam after 8 d) on 2 % malt agar, with white aerial mycelium growing in fan-like waves across the plate. Conidiomata matured after 4–5 weeks incubation and conidia were aseptate, hyaline and ovoid, measuring $4\text{--}8 \times 1.5\text{--}2.5 \mu\text{m}$ (mean $6 \times 2 \mu\text{m}$). Perithecia of *Gnomonia intermedia*, with one or multiple beaks, developed in vitro on the surface of isolated colonies of *D. betulina* and were usually clustered around the original piece of host tissue. Asci measured $30\text{--}43 \times 6\text{--}12 \mu\text{m}$ (mean $35 \times 8 \mu\text{m}$), and ascospores (Fig 1A) were one-septate, hyaline and ellipsoid, measuring $9\text{--}14 \times 2 \mu\text{m}$ (mean $12 \times 2 \mu\text{m}$). Ascospores germinated well on 2 % malt agar, and consistently developed into colonies that were morphologically identical to those of *D. betulina*, producing conidia (Fig 1B) typical of *D. betulina*.

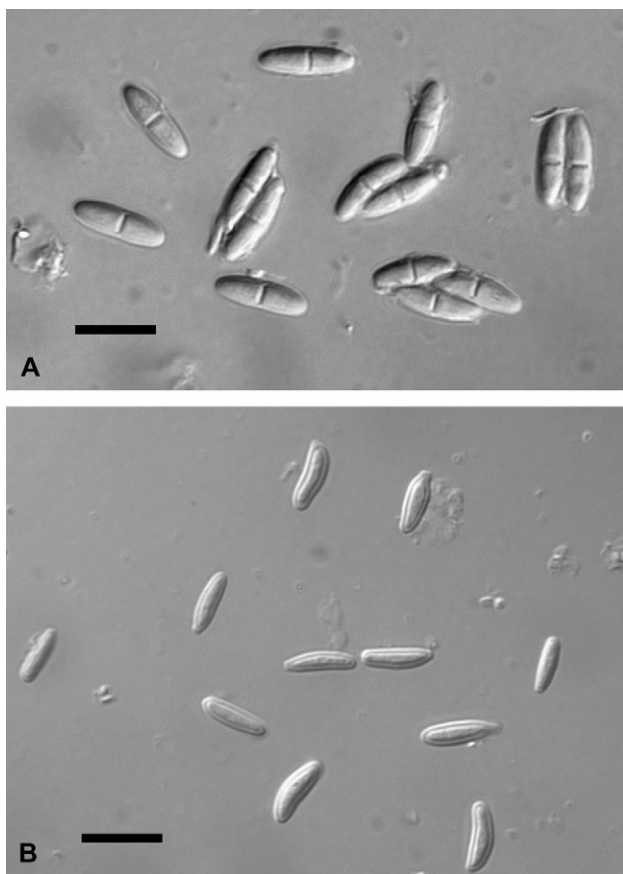


Fig 1 – A. Ascospores of *Gnomonia intermedia*, specimen BPI 611453 (Rehm Ascomycetes Fas. 42, No. 1794). Scale bar = 10 μm . **B.** *Discula betulina* conidia produced on PDA, isolate 2048a (CBS 119194). Scale bar = 10 μm .

Perithecia of *G. intermedia* developed on *D. betulina*-infected leaves of silver and downy birch that were overwintered either outside or on wet filter paper at 4 °C. Single ascospore isolates developed into typical *D. betulina* colonies. Perithecia of *G. intermedia* did not develop on leaves maintained on dry filter paper at 4 °C. Perithecia of *G. setacea* and pseudothecia of *Venturia ditricha* were also commonly found on leaves overwintered either outside or on wet filter paper at 4 °C.

Isolate crosses

Fertile perithecia of *Gnomonia intermedia*, containing mature ascospores, were produced in 50 out of the 210 different crosses among the 20 isolates of *Discula betulina* and one isolate of *G. intermedia* (Table 2). Perithecia developed in 21 crosses within two months of inoculation, in a further 26 crosses after four months and in another three more crosses after six months; the experiment was then terminated. Perithecia formed either on the toothpick or adjacent to it where mycelium from the two parental isolates met. All 21 isolates were able to form fertile perithecia in at least one cross, and seven isolates were able to form fertile perithecia in seven to ten different crosses (Table 2). The 21 isolates could be grouped into two sexual compatibility types based on these results, consisting of 13 and eight isolates, respectively (Table 2). All single ascospore isolates developed into colonies that were morphologically identical to *D. betulina*, producing typical *D. betulina* conidia.

Pathogenicity tests

Lesions similar to those observed on young shoots in the field developed on the leading shoots of all seedlings inoculated with the four isolates. Mean lesion lengths \pm s.e. at 14 d after inoculation were 21 ± 1.7 mm and 25 ± 2.1 mm for the two isolates of *Discula betulina*, and 18 ± 2.6 mm and 13 ± 1.4 mm for the two isolates of *Gnomonia intermedia*. After 14 d, lesions started to girdle shoots, causing wilting and die-back. Control seedlings did not develop lesions. In the tests for inoculum viability, all mycelial test plugs developed into colonies typical of each respective isolate, indicating 100 % inoculum viability. In back-isolations made from the inoculated seedlings, colonies of *D. betulina* grew from all tissue pieces plated for the four isolates, but from none of the controls.

DNA analysis

ITS regions 1 and 2 were sequenced for four isolates of *Gnomonia intermedia* and five isolates of *Discula betulina*. Of the 574 base pairs (bp) sequenced using the ITS 5 and ITS 4 primers, only three bp differed among the isolates. A GenBank BLAST search with isolate 2048a returned *Sirococcus clavignenti-juglandacearum* as the closest match with identities = 410/420 (97 %) and gaps = 3/420 (0 %). Analysis of the entire ITS 1 and 2 regions for *S. clavignenti-juglandacearum* found that of the 542 alignment positions in common, the two taxa differed by four gapped positions, 12 transversions, and six transitions with a total of 518/542 identities (95 %). *Gnomoniella nana* from dwarf birch was found to be even closer and differed by six gapped positions and three base substitutions for a total

Table 2 – Results of crossing of isolates of *Discula betulina* and *Gnomonia intermedia*

| | 2013a | 2019 | 2024 | 2041a | 2004 | 2078 | GAA15 | 2072 | 2048a | 2032 | 2005a | 2006a | 2044a | 2043a | 2080 | 2075 | 2076 | 2077 | 2071 | 2073 | 2081 |
|-------|-------|------|------|-------|------|------|-------|------|-------|------|-------|-------|-------|-------|------|------|------|------|------|------|------|
| 2013a | | | | | | | | | | | | | | | | + | | + | | | + |
| 2019 | | | | | | | | | | | | | | | + | + | | + | + | | + |
| 2024 | | | | | | | | | | | | | | | + | + | | + | + | | + |
| 2041 | | | | | | | | | | | | + | | | + | + | | + | + | + | + |
| 2004 | | | | | | | | | | + | | | | | + | + | | + | + | | + |
| 2078 | | | | | | | | | | + | + | + | + | + | + | + | + | | + | | |
| GAA15 | | | | | | | | | + | | + | + | + | | + | + | + | + | + | + | + |
| 2072 | | | | | | | | | | | | | | | + | + | | | + | | + |
| 2048a | | | | | | | + | | | | | | | | | | | | | | |
| 2032 | | | | | + | + | | | | | | | | | | | | | | | |
| 2005a | | | | | | + | + | | | | | | | | | | | | | | |
| 2006a | | | | + | | + | + | | | | | | | | | | | | | | |
| 2044a | | | | | | + | + | | | | | | | | | | | | | | |
| 2043a | | | | | | + | | | | | | | | | | | | | | | |
| 2080 | | + | + | + | + | + | + | + | | | | | | | | | | | | | |
| 2075 | + | + | + | + | + | + | + | + | | | | | | | | | | | | | |
| 2076 | | | | | | + | + | | | | | | | | | | | | | | |
| 2077 | + | + | + | + | + | | + | | | | | | | | | | | | | | |
| 2071 | | + | + | + | + | + | + | + | | | | | | | | | | | | | |
| 2073 | | | | + | | | + | | | | | | | | | | | | | | |
| 2081 | + | + | + | + | + | | + | + | | | | | | | | | | | | | |

The production of fertile perithecia in any pairwise cross is indicated by +. The two mating types are indicated by shaded and non-shaded areas.

of 571/579 identities (98 %). *Gnomoniella nana* shared one substitution with five isolates of *Gnomonia intermedia*/D. *betulina*. Percent identities between *G. intermedia* 2048a and other representative taxa in the *Gnomoniaceae* are summarised in Table 3.

Phylogenetic analysis of the LSU and ITS sequences of *G. intermedia* 2048a with representatives of the *Gnomoniaceae* and *Melanconidaceae*, using the *Cryphonectria* complex as out-group taxa, resulted in two equally parsimonious trees that did not differ in overall topology (length = 496, CI = 0.681, RI = 0.714, RC = 0.487, HI = 0.319). One tree is shown in Fig 2 with MP BS above and NJ BS below the branches. BSs less than 70 % were not reported. As found with ITS BLAST analyses, *Gnomoniella nana* and *S. clavignenti-juglandacearum* were the closest relatives to *Gnomonia intermedia*. In addition *G. leptostyla* was supported (70 % MP, 76 % NJ) as related to these three taxa. *G. gnomon*, the type species of *Gnomonia* is not closely related to these taxa, nor is *Gnomoniella fraxini*, although it is not the type species of *Gnomoniella*.

Discussion

Ascomyces of *Gnomonia intermedia* consistently develop into colonies morphologically identical to those of *Discula betulina* and produce typical D. *betulina* conidia, which indicate that

Table 3 – Percent identities between *Gnomonia intermedia* isolate 2048a and other representative taxa in the *Gnomoniaceae*

| Pairwise comparisons | Identities | Gapped positions | Percent identity |
|--|------------|------------------|------------------|
| <i>Gnomonia intermedia</i> versus <i>Sirococcus clavignenti-juglandacearum</i> | 518/542 | 4/542 | 95 % |
| <i>G. intermedia</i> versus <i>Gnomoniella nana</i> | 571/579 | 6/579 | 98 % |
| <i>G. intermedia</i> versus <i>G. gnomon</i> | 503/557 | 19/557 | 90 % |
| <i>G. intermedia</i> versus <i>Gnomoniella fraxini</i> | 490/546 | 19/546 | 89 % |
| <i>G. intermedia</i> versus <i>Discula umbrinella</i> | 498/544 | 21/544 | 91 % |
| <i>G. intermedia</i> versus <i>D. destructiva</i> | 517/568 | 18/568 | 91 % |
| <i>S. clavignenti-juglandacearum</i> versus <i>S. conigenus</i> | 475/550 | 16/550 | 86 % |
| <i>D. umbrinella</i> versus <i>D. destructiva</i> | 496/528 | 11/528 | 93 % |

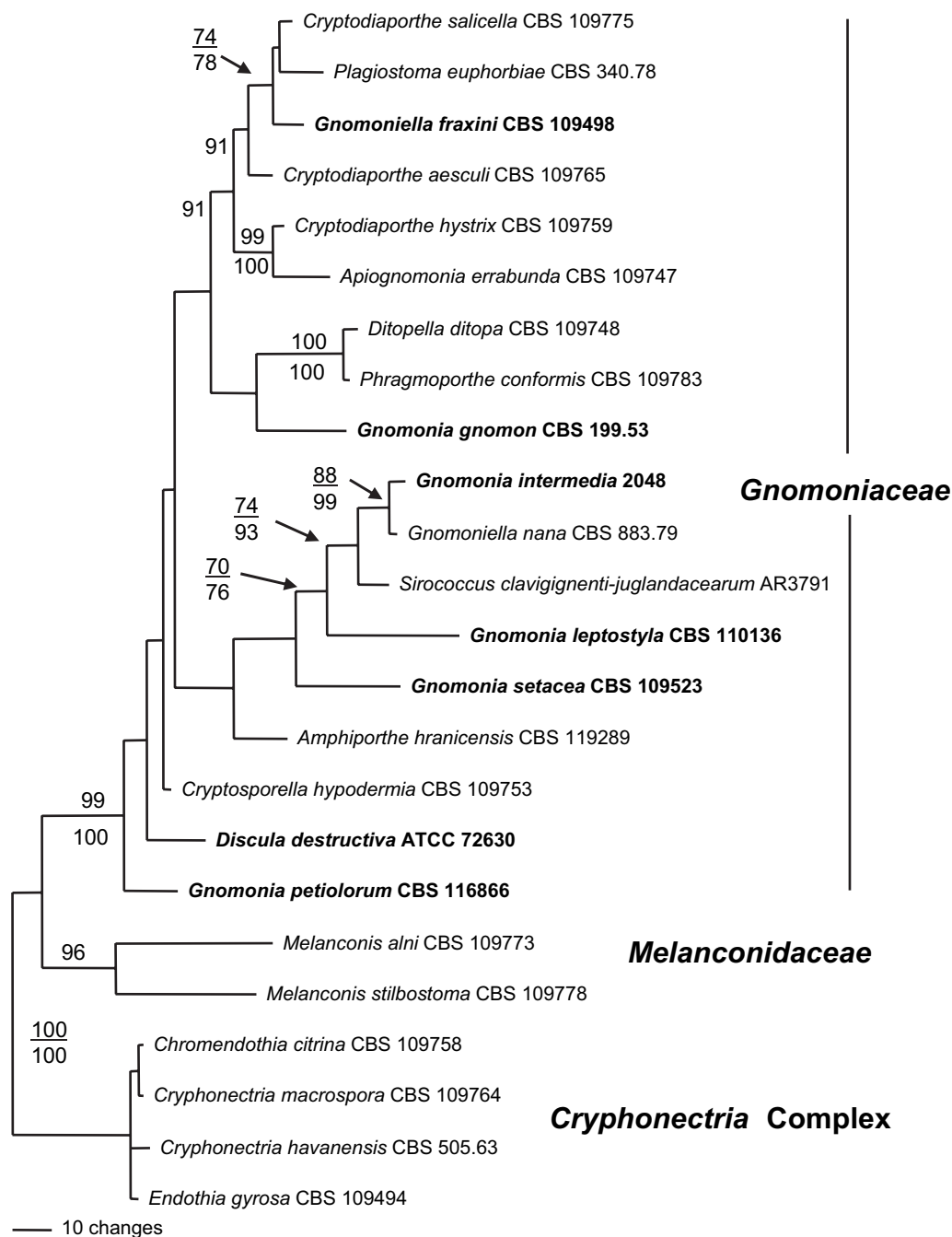


Fig 2 – MP phylogenetic analysis of LSU and ITS nu-rDNA for representative taxa in the Gnomoniaceae. BS supports 70 % or greater are above (MP) and below (NJ) the branches. Strains are identified with strain number and LSU sequences not generated in this study are from [Castlebury et al. \(2002\)](#). ITS sequences for *Gnomonia gnomon* and *G. setacea* are from [Sogonov et al. \(2005\)](#) and for *Apiognomonia errabunda* from GenBank accession [DQ313525](#). Species of *Gnomonia*, *Gnomoniella* and *Discula* are in bold type.

D. betulina and *G. intermedia* are two states of the same species. Measurements of conidia and ascospores in this study agreed with previous descriptions for these two fungi ([Barr 1978](#); [Phillips & Burdekin 1982](#)), although asci were slightly larger than those described by [Barr \(1978\)](#).

All 21 isolates of *D. betulina* were able to produce fertile perithecia of *G. intermedia* when mated, suggesting that this fungus is heterothallic with an outcrossing breeding system, although isolates differed in their ability to mate successfully

with isolates of the opposite mating type. The isolates of *D. betulina* fell into the two sexual compatibility groups in fairly equal numbers, indicating a single locus for mating type with two alleles. This mating system is typical for many species within the ascomycetes ([Correll & Gordon 1999](#)).

In Scotland, *G. intermedia* has not been found on birch during the summer, but developed *in vitro* from isolations conducted during late-summer and autumn, although not during early summer ([Green 2004](#)). In this study, perithecia

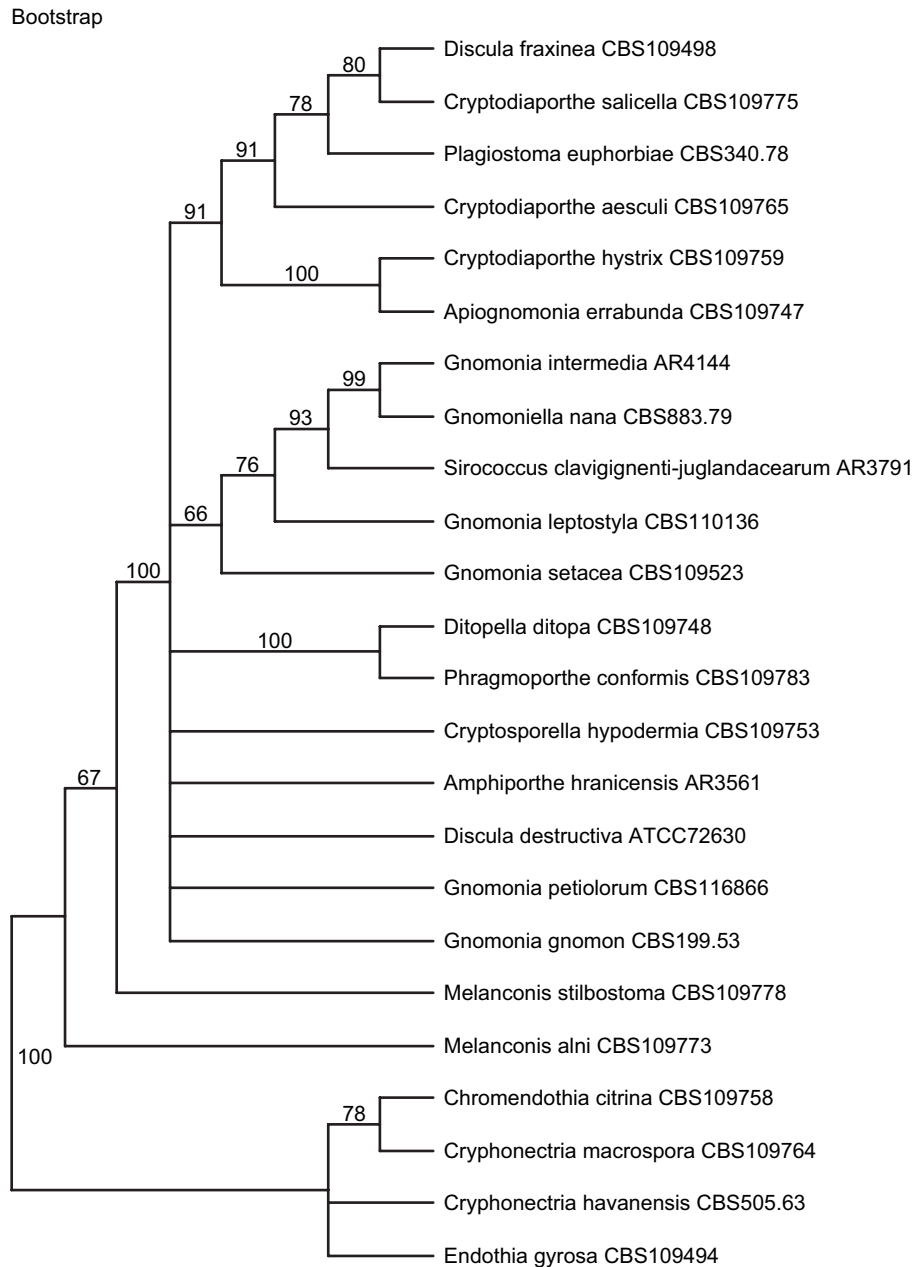


Fig 2 (continued)

of *G. intermedia* formed abundantly on leaves of silver and downy birch that were naturally infected with *D. betulina* and kept in moist conditions over winter. Barr (1978) also described *G. intermedia* as occurring on overwintered birch leaves. These observations suggest that the perithecial state is important for survival of this fungus over winter, although the role of ascospores in initiating disease or endophytic infections during the early growing season has yet to be confirmed.

Pathogenicity tests on downy birch demonstrated that *G. intermedia* and *D. betulina* caused lesions that expanded rapidly to girdle shoots, resulting in die-back. Lesions caused by *D. betulina* were slightly longer than those caused by *G. intermedia*, but further inoculation studies are required to

determine whether conidial and ascospore-derived isolates differ in their aggressiveness on birch. The consistent re-isolation of *D. betulina* from lesions caused by both single-conidial and single-ascospore isolates provides further evidence to support our conclusion that *D. betulina* is the anamorph of *G. intermedia*. *D. betulina*, *Marssonina betulae* and *Septoria betulae* appear to be dominant leaf spot pathogens on young, planted birch in Scotland (Green 2004, 2005a), and often occur together at the same sites. The three diseases can be distinguished in the field by leaf lesion characteristics, with *D. betulina* forming dark-brown lesions with more definite margins than lesions caused by *M. betulae*, which are distinctly stellate. Lesions caused by *S. betulae* are considerably smaller and more circular than those caused by the other two fungi, and are ochre to

pale brown with darker margins (Green 2005a). Teleomorph states have not been described for *M. betulae* or *S. betulae* and there is particular interest in determining whether a teleomorph state exists for *M. betulae* in Scotland where it has been shown to be an aggressive pathogen on young birch trees (Green 2005b). Recent progress has been made in determining the relationships between other leaf spot fungi on birch, for example, in Finland the birch leaf spot pathogen *Pyrenopeziza betulicola* has been linked with a species of *Cylindrosporium* (Paavolainen et al. 2000). To date, *P. betulicola* is absent from literature reporting the fungal associations of birch in the UK, although a *Cylindrosporium*-type fungus similar to that described by Paavolainen et al. (2000) has been found causing leaf spots on birch in Scotland during summer 2006 (S.G., unpubl.).

Results from the ITS sequencing confirm that *D. betulina* isolated from silver birch is the same species as *G. intermedia* and that it is closely related to but seemingly distinct from an isolate of *G. nana* from dwarf birch in Finland (CBS 883.79). In addition, *G. intermedia* is closely related to the butternut canker fungus, *Sirococcus clavigignenti-juglandacearum*. Butternut canker is a serious pathogen of *Juglans cinerea* in eastern North America and is assumed to have been introduced into North America prior to 1967 (Furnier et al. 1999).

The taxonomy of both teleomorph and anamorph genera in the *Gnomoniaceae* is somewhat confused. Ascospore and perithecial characters are currently used to delimit genera (Barr 1978; Monod 1983). However, as molecular data have become available it is apparent that many genera are not monophyletic, including the genus *Gnomonia* (Castlebury et al. 2002; Sogonov et al. 2005). In the case of *Gnomonia* and *Gnomoniella*, both produce scattered perithecia with beaks protruding from leaves of many deciduous trees and they have been distinguished by the production of septate ascospores in *Gnomonia* and aseptate ascospores in *Gnomoniella* (Barr 1978; Monod 1983). In this case, *G. intermedia* and *Gnomoniella nana* appear to be sister species and neither are particularly closely related to the type species of *Gnomonia*, *G. gnomon*, or *Gnomoniella fraxini*, which has a *Discula amamorph*, *D. fraxinea*. The type species of the genus *Sirococcus* is *S. conigenus* (Cannon & Minter 1983) and a teleomorph has not been reported for this fungus. ITS sequences available in GenBank suggest that *S. clavigignenti-juglandacearum* is not closely related to *S. conigenus* with only 86 % identities between the two taxa. It is likely that as more information is obtained about this group of fungi, *Gnomonia intermedia* and *D. betulina* as well as *G. nana* and *S. clavigignenti-juglandacearum* will be transferred from their current generic placements into a single phylogenetic genus.

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